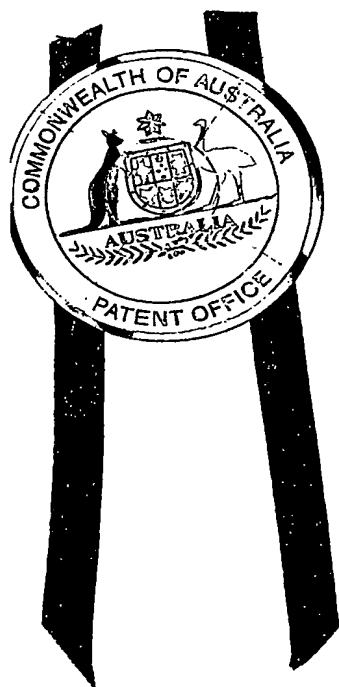REC'D  0 2 JUL 2003

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in connection with Application No. PS 2826 for a patent by GARVAN
INSTITUTE OF MEDICAL RESEARCH as filed on 07 June 2002.

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# AUSTRALIA

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## Garvan Institute of Medical Research

## PROVISIONAL  SPECIFICATION

*Invention Title:*

*Method of inhibiting cell proliferation*

The invention is described in the following statement:

# *Method of inhibiting cell proliferation*

## Field of the Invention

The present invention relates to a method inhibiting proliferation of
5   prostate cancer cells, such as in a method of treating prostate cancer.

## Background of the invention

Prostate cancer occurs frequently in men, is currently the second most
common cause of cancer-related death and incidence is growing. Our
10   understanding of its aetiology is limited and unlike certain other cancers, little
progress has been made in elucidating its cause. Efforts have been made to
identify genes responsible for familial prostate cancer. At least seven
chromosomal loci have been reported, however the genes responsible for
prostate cancer in all these loci have not yet been identified. Although an
15   inherited genetic predisposition occurs in only 5-10% of cases, it is possible
that identification of germline mutations may shed light on sporadic cases as
both forms share the same histopathological features. The majority of
researchers have focused on somatic defects in sporadic prostate cancer.
Classical cytogenetic studies are difficult to apply to solid tumours and so far no
20   consistent chromosomal changes have been observed. Although comparative
genome hybridisation and loss of heterozygosity analysis have shown both
gain and loss of genomic DNA, the majority of genes involved are still
unknown. Oncogenes and tumour suppressor genes known to be associated
with other malignancies have a remarkably low frequency of mutation or
25   deletion in prostate cancer. Using technologies that compare the steady-state
mRNA levels between normal and cancerous prostate, a list of genes have
been revealed to be either over or underexpressed in prostate cancer tissue or
cell-lines. Although proteomics and tissue array approaches are now being
used, relatively few genes have yet been verified to be differentially expressed
30   in a reasonable number of specimens at the protein level. Direct evidence for
the importance of these differentially expressed genes in prostate cancer

initiation is lacking. As a result, although progress is rapid, the application of this new knowledge in controlling mortality and morbidity from prostate cancer is slow at present.

Emerging evidence from epidemiological studies indicates a strong
5 association between prostate cancer risk and total fat intake (Kolonel et al., 1999 J. Natl Cancer Inst. 91: 414), although the biochemical link between dietary lipids and genesis of prostate cancer remains unclear. Previous studies have demonstrated that both cyclooxygenase (COX) and lipoxygenase (LOX) products of arachidonic acid metabolism, the prostaglandins (PG), and
10 hydroxyeicosatetraenoic acids (HETEs) respectively, contribute to formation and/or progression of prostate cancer. They are implicated in promotion of tumour cell proliferation, motility, invasion and metastasis, and induction of angiogenesis both *in vitro* and in animal models. Interestingly, arachidonic acid levels are lower in malignant than benign (BPH) prostate tissue while PG and
15 HETE synthesis from labelled arachidonic acid is significantly increased. However, the activity of arachidonic acid mobilising enzymes phospholipase A2 (PLA2) and fatty acyl-CoA lysophosphatidylcholine acyltransferase, are also increased, suggesting an increased flux of arachidonic acid through the COX and LOX pathways.

20 sPLA$_2$-IIA is elevated in prostate cancer (Graff et al., 2001, Clin. Cancer Res. 7: 3857-3861; Jiang et al., 2002, Am. J. Pathol. 160: 667-671) and enhanced sPLA$_2$-IIA expression has been inversely related to 5-year patient survival (Graff et al., 2001). In addition, the chromosomal location of several sPLA2 genes including sPLA$_2$-IIA (1p35-ter), overlaps with one prostate cancer
25 susceptibility locus CAPB (Nwosu et al., 2001, Human Mol. Genet. 10: 2313-2318).

Summary of the invention

We have now found that treatment of prostate cancer cells with
30 sPLA$_2$-IIA potently increases proliferation of the cells. This provides evidence

for the first time of the direct role of sPLA$_2$-IIA in the proliferation of prostate cancer cells and highlights the enzyme as an important therapeutic target.

We have also shown that the potent proliferative effect of sPLA$_2$-IIA is blocked by the addition of a conformationally constrained peptide based on 5 amino acid residues 70-74 of the native sPLA$_2$-IIA protein.

Accordingly, in a first aspect the present invention provides a method of inhibiting or reducing the proliferation of prostate cancer cells, the method comprising administering to the cells a conformationally constrained molecule derived from a peptide consisting essentially of amino acid residues 70 to 74 of 10 a human sPLA$_2$-IIA protein, or the equivalent residues in other sPLA$_2$-IIA proteins.

In a further aspect the present invention provides a method for the treatment of prostate cancer, the method comprising administering to a subject in need thereof a conformationally constrained molecule derived from a peptide 15 consisting essentially of amino acid residues 70 to 74 of a human sPLA$_2$-IIA protein, or the equivalent residues in other sPLA$_2$-IIA proteins.

In yet a further aspect the present invention provides the use of a conformationally constrained molecule derived from a peptide consisting essentially of amino acid residues 70 to 74 of a human sPLA$_2$-IIA protein, or the 20 equivalent residues in other sPLA$_2$-IIA proteins, in the manufacture of a medicament for the treatment of prostate cancer.

Preferably, the conformationally constrained molecule is a peptide, more preferably a cyclic peptide.

In a preferred embodiment, the conformationally constrained peptide is a 25 cyclic peptide of the following formula:

A1-A2-A3-A4-A5

in which

A1 is F or Y or W or 2Nap

A2 is L or I

30 A3 is S or T

A4 is F or Y or W or 2Nap

A5 is R or K.

In a further preferred embodiment of the present invention, the peptide is selected from the group consisting of cFLSYK, cFLSYR and c(2NapA)LS(2NapA)R.

5    When used herein the term "cFLSYK" means "cyclic FLSYK", "cFLSYR" means "cyclic FLSYR" and "c(2NapA)LS(2NapA)R" means "cyclic (2Nap)LS (2Nap)R". The term "2NapA" is an abbreviation for 2-naphthylalanine.

In a further preferred embodiment, the peptide comprises D-amino acids and has a sequence which corresponds to the reverse sequence of a peptide

10   according to the first aspect of the present invention.


## Brief description of the Figures


Figure 1: Graph showing that sPLA$_2$-IIA significantly enhances LNCaP

15   proliferation. Data represent the mean +/- SD of duplicate experiments performed in quadruplicate.  Control OD (490 nm) was 0.34 +/- 0.02.  * p$\leq$0.05 vs control (1-way ANOVA).


Figure 2:   Graph showing suppression of proliferation in LNCaP cells by

20   c(2Nap)LS(2Nap)R..  Data are mean +/- SD of quadruplicate determinations from one experiment. *. p$\leq$ 0.05 vs Control, (1-way ANOVA).


## Detailed description of the invention

Unless defined otherwise, all technical and scientific terms used herein

25   have the same meaning as commonly understood by one of ordinary skill in the art (e.g. in cell culture, molecular genetics, nucleic acid chemistry, hybridization techniques and biochemistry). Standard techniques are used for molecular, genetic and biochemical methods (see generally, Sambrook et al., Molecular Cloning: A Laboratory Manual, 2nd ed. (1989) Cold·Spring Harbor Laboratory

30   Press, Cold Spring Harbor, N.Y. and Ausubel et al., Short Protocols in Molecular Biology (1999) 4th Ed, John Wiley & Sons, Inc. - and the full version

entitled Current Protocols in Molecular Biology, which are incorporated herein by reference) and chemical methods.

Throughout this specification the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a

5    stated element, integer or step, or group of elements, integers or steps, but not the exclusion of any other element, integer or step, or group of elements, integers or steps.

*Peptides and peptide analogues*

10       The methods of the present invention involve the administration of conformationally constrained molecules derived from a peptide consisting essentially of amino acid residues 70 to 74 of a human sPLA2-IIA protein, or the equivalent residues in other sPLA$_2$-IIA proteins.

In general, reference to amino acid residues 70 to 74 of the human

15    sPLA$_2$-IIA protein is taken to include reference to the equivalent residues in other sPLA$_2$-IIA proteins, such as orthologues of human sPLA$_2$-IIA.

The term "conformationally constrained molecules" means conformationally constrained peptides and conformationally constrained peptide analogues and derivatives.

20       Thus the conformationally constrained molecules according to the present invention include conformationally constrained peptides consisting essentially of residues 70 to 74 of the human sPLA2-IIA protein, and analogues and derivatives thereof.

The term "analogues" refers to molecules having a chemically analogous

25    structure to the naturally occurring alpha-amino acids present as residues 70 to 74 of the human sPLA$_2$-IIA protein. Examples include molecules containing *gem*-diaminoalkyl groups or alklylmalonyl groups.

The term "derivatives" includes alpha amino acids wherein one or more side groups found in the naturally occurring alpha-amino acids present as

30    residues 70 to 74 of human sPLA$_2$-IIA protein have been modified. Thus, for example the naturally-occurring amino acids present in residues 70 to 74 of the

human sPLA$_2$-IIA protein may be replaced with a variety of uncoded or modified amino acids such as the corresponding D-amino acid or N-methyl amino acid. Other modifications include substitution of hydroxyl, thiol, amino and carboxyl functional groups with chemically similar groups.

5  The present invention encompasses the use of conformationally constrained peptidomimetics of the biologically active human sPLA$_2$-IIA peptide (amino acid residues 70 to ), i.e. analogues and derivatives which mimic the activity of said peptide and are therefore capable of inhibiting the sPLA$_2$-IIA dependent proliferation of prostate cancer cells. These peptidomimetics are

10  preferably substantially similar in both three-dimensional shape and biological activity to the specific sPLA$_2$-IIA peptides described herein. Substantial similarity means that the geometric relationship of groups in the peptide that react with the sPLA2-IIa enzyme is preserved and at the same time, that the peptidomimetic will inhibit the sPLA$_2$-IIA dependent proliferation of prostate

15  cancer cells.

A peptidomimetic is a molecule that mimics the biological activity of a peptide but is no longer peptidic in chemical nature. By strict definition, a peptidomimetic is a molecule that no longer contains any peptide bonds (that is, amide bonds between amino acids). However, the term peptide mimetic is

20  sometimes used to describe molecules that are no longer completely peptidic in nature, such as pseudo-peptides, semi-peptides and peptoids. Whether completely or partially non-peptide, peptidomimetics for use in the methods of the invention provide a spatial arrangement of reactive chemical moieties that closely resembles the three-dimensional arrangement of active groups in the

25  peptide on which the peptidomimetic is based. As a result of this similar active-site geometry, the peptidomimetic has effects on biological systems which are similar to the biological activity of the peptide.

There are clear advantages for using a mimetic of a given peptide rather than the peptide itself, because peptides commonly exhibit two undesirable

30  properties: (1) poor bioavailability; and (2) short duration of action. Peptide mimetics offer an obvious route around these two major obstacles, since the

molecules concerned are small enough to be both orally active and have a long duration of action. There are also considerable cost savings and improved patient compliance associated with peptide mimetics, since they can be administered orally compared with parenteral administration for peptides.

5 Furthermore, peptide mimetics are much cheaper to produce than peptides.

Suitable peptidomimetics based on residues 70 to 74 of human sPLA$_2$-IIA peptides and having similar biological activities, and therefore similar therapeutic utilities, can be developed using readily available techniques. Thus, for example, peptide bonds can be replaced by non-peptide bonds that

10 allow the peptidomimetic to adopt a similar structure, and therefore biological activity, to the original peptide. Further modifications can also be made by replacing chemical groups of the amino acids with other chemical groups of similar structure. The development of peptidomimetics derived from sPLA$_2$-IIA peptides based on residues 70 to 74 of human sPLA$_2$-IIA can be aided by

15 determining the tertiary structure of the original peptide by NMR spectroscopy, crystallography and/or computer-aided molecular modelling. These techniques aid in the development of analogues/derivatives of higher potency and/or greater bioavailability and/or greater stability than the original peptide (Dean, 1994, BioEssays, 16: 683-687; Cohen and Shatzmiller, 1993, J. Mol. Graph.,

20 11: 166-173; Wiley and Rich, 1993, Med. Res. Rev., 13: 327-384; Moore, 1994, Trends Pharmacol. Sci., 15: 124-129; Hruby, 1993, Biopolymers, 33: 1073-1082; Bugg et al., 1993, Sci. Am., 269: 92-98.

Information on the structure of an sPLA2-IIA peptide consisting essentially of residues 70 to 74 of human sPLA2-IIA can be used to search

25 three-dimensional databases to identify molecules having a similar structure, using programs such as MACCS-3D and ISIS/3D (Molecular Design Ltd., San Leandro, CA), ChemDBS-3D (Chemical Design Ltd., Oxford, U.K.), and Sybyl/3DB Unity (Tripos Associates, St. Louis, MO).

Databases of chemical structures are available from a number of

30 sources including Cambridge Crystallographic Data Centre (Cambridge, U.K.),

Chemical Abstracts Service (Columbus, OH), and ACD-3D (Molecular Design Ltd).

*De novo* design programs include Ludi (Accelrys), Leapfrog (Tripos Associates) and Aladdin (Daylight Chemical Information Systems, Irvine, CA).

5      Those skilled in the art will recognize that the design of a mimetic may require slight structural alteration or adjustment of a chemical structure designed or identified using these databases.

Peptide derivatives and peptidomimetic compounds based on amino acid residues 70 to 74 of human $sPLA_2$-IIA can be tested to determined whether 10   they are capable of inhibiting $sPLA_2$-IIA dependent proliferation of prostate cancer cells using the assay described herein. Preferred peptide derivatives and peptidomimetics have at least 90%, preferably at least the same anti-proliferative activity toward prostate cancer cells as cFLSYR. It is also preferred that peptide derivatives and peptidomimetics specifically inhibit 15   $sPLA_2$-IIA and not other sPLA enzymes such as $sPLA_2$-IB.

The molecules, such as peptides, used in the methods of the present invention are conformationally constrained. Conformational constraint refers to the stability and preferred conformation of the three-dimensional shape assumed by a peptide. Conformational constraints include local constraints, 20   involving restricting the conformational mobility of a single residue in a peptide; regional constraints, involving restricting the conformational mobility of a group of residues, which residues may form some secondary structural unit; and global constraints, involving the entire peptide structure.

The active conformation of a peptide may be stabilized by a covalent 25   modification, such as cyclization or by incorporation of gamma-lactam or other types of bridges. For example, side chains can be cyclized to the backbone so as create a L-gamma-lactam moiety on each side of the interaction site. See, generally, Hruby *et al.*, 1992, "Applications of Synthetic Peptides," in Synthetic Peptides: A User's Guide: 259-345 (W. H. Freeman & Co.). Cyclization also 30   can be achieved, for example, by formation of cystine bridges, coupling of amino and carboxy terminal groups of respective terminal amino acids, or

coupling of the amino group of a Lys residue or a related homolog with a carboxy group of Asp, Glu or a related homolog. Coupling of the alpha-amino group of a polypeptide with the epsilon-amino group of a lysine residue, using iodoacetic anhydride, can be also undertaken. See, for example, Wood and

5 Wetzel, 1992, *Int'l J. Peptide Protein Res.* 39: 533-39.

Another approach described in US 5,891,418 is to include a metal-ion complexing backbone in the peptide structure. Typically, the preferred metal-peptide backbone is based on the requisite number of particular coordinating groups required by the coordination sphere of a given complexing metal ion. In

10 general, most of the metal ions that may prove useful have a coordination number of four to six. The nature of the coordinating groups in the peptide chain includes nitrogen atoms with amine, amide, imidazole, or guanidino functionalities; sulfur atoms of thiols or disulfides; and oxygen atoms of hydroxy, phenolic, carbonyl, or carboxyl functionalities. In addition, the peptide

15 chain or individual amino acids can be chemically altered to include a coordinating group, such as for example oxime, hydrazino, sulfhydryl, phosphate, cyano, pyridino, piperidino, or morpholino.

A further approach approach is to use bifunctional crosslinkers, such as N-succinimidyl 3-(2 pyridyldithio) propionate, succinimidyl 6-[3-(2 pyridyldithio)

20 propionamido] hexanoate, and sulfosuccinimidyl 6-[3-(2 pyridyldithio) propionamido]hexanoate (see US Patent 5,580,853).

Techniques for chemically synthesising the peptides and derivatives described above are described in the above references and also reviewed by Borgia and Fields, 2000, *TibTech* 18: 243-251 and described in detail in the

25 references contained therein.


*Pharmaceutical compositions and administration*

We have shown that administration of exogenous sPLA$_2$-IIA to prostate cells stimulates cell proliferation. We have also shown that administration of a

30 conformationally constrained cyclic peptide consisting of residues 70 to 74 of human sPLA$_2$-IIA, or a derivative of said peptide, inhibits sPLA$_2$-IIA mediated

cell proliferation. Consequently, the conformationally constrained molecules according to the present invention can be used to inhibit or reduce prostate cell proliferation in cells, particularly in cells with elevated sPLA-IIA activity such as prostate cancer cells.

5       The peptides, analogues and derivatives thereof described above may preferred be combined with various components to produce compositions. Preferably the compositions are combined with a pharmaceutically acceptable carrier or diluent to produce a pharmaceutical composition (which may be for human or animal use). Suitable carriers and diluents include isotonic saline

10 solutions, for example phosphate-buffered saline, water, dry powders and micelles. The composition may be administered by any means known in the art. Modes of delivery include, but are not limited to, direct injection, topical delivery (e.g. by atomised nasal delivery or nasal drops) or oral delivery. Accordingly, the composition may be formulated, *inter alia*, for topical,

15 parenteral, intramuscular, intravenous, subcutaneous, intraocular, oral or transdermal administration.

      Typically, each peptide or analogue or derivative thereof may be administered at a dose of from 0.01 to 30 mg/kg body weight, preferably from 0.1 to 10 mg/kg, more preferably from 0.1 to 1 mg/kg body weight.

20       The routes of administration and dosages described are intended only as a guide since a skilled practitioner will be able to determine readily the optimum route of administration and dosage for any particular patient and condition.

      The present invention will now be described further with reference to the following examples which are intended to be illustrative only and non-limiting.

25

## EXAMPLES

### Example 1: Effect of sPLA$_2$-IIA on proliferation of prostate cells

      Prostate cancer cells (LNCaP) Cells were seeded at 5000 cells/well in

30 96-well plates in RPMI 5% FCS and treated with either 1 nM androgen or various doses of sPLA$_2$-IIA. After 4 days, cells were treated for 72 hours in

media containing charcoal-stripped FCS. Proliferation was measured by MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium) assay.

sPLA$_2$-IIA potently increased the proliferation of LNCaP cells as
5    indicated by both cell counts and MTS proliferation assays. The lowest dose of sPLA$_2$-IIA (0.4 nM) showed a clear effect on cell proliferation and was comparable to androgen (1 nM) stimulation (see Figure 1).

These results indicate for the first time that enhanced sPLA$_2$-IIA activity can promote proliferation in prostate cancer cells and provide evidence of an
10   oncogenic role of sPLA$_2$-IIA in prostate cancer.


Example 2: Effect of peptide inhibitors on sPLA2 dependent proliferation of prostate cells

We have shown previously that human sPLA$_2$-IIA is dose-dependently
15   inhibited by a pentapeptide sequence consisting of residues 70 to 74 of the native sPLA$_2$-IIA (Tseng et al., 1996, J. Biol. Chem. 271: 23992). However, because of the inherent flexibility of the linear peptide sequence, inhibition was weak in *in vitro* activity assays. We have therefore recently used analogue screens, molecular docking experiments and peptide-protein interaction
20   analysis approaches to characterise the structural features of the peptide which are important for inhibition. With this information we have designed more potent peptide-based inhibitors and characterised the mechanism of interaction of the peptide with the enzyme (Church et al., 2001, J. Biol. Chem. 276: 33156). These studies have identified two cyclic peptides, cFLSYR and a
25   cyclic peptide where F and Y are substituted with 2-naphthylalanine (c(2NapA)LS(2NapA)R), which show significant improvement in potency over linear peptides in an *in vitro* activity assay. The potency of inhibition was also reflected in steady-state binding to sPLA$_2$-IIA as determined by surface plasmon resonance. Binding of the most potent inhibitor (c(2Nap)LS(2Nap)R)
30   was specific in that the compound does not bind to sPLA$_2$-IB or to murine immunoglobulin.

We therefore sought to determine whether these compounds would block $sPLA_2$-IIA mediated proliferation in prostate cancer cells using the assay described in Example 1.

To determine if the peptide inhibitors would block $sPLA_2$-IIA-dependent
5   proliferation, we treated LNCaP cells with c(2Nap)LS(2Nap)R in the presence and absence of $sPLA_2$-IIA under similar conditions to those used in Example 1. As shown in Figure 2, the cyclic peptide potently blocks $sPLA_2$-IIA-dependent proliferation and importantly also reduces the MTS response of unstimulated cells, suggesting that LNCaP cell growth is modulated in part by the presence
10  of endogenous $sPLA_2$-IIA.

All publications mentioned in the above specification are herein incorporated by reference. Various modifications and variations of the described methods and system of the invention will be apparent to those skilled in the art without departing from the scope and spirit of the invention. Although
15  the invention has been described in connection with specific preferred embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the described modes for carrying out the invention which are apparent to those skilled in molecular biology or related fields are intended to be within the
20  scope of the invention.


Dated this seventh day of June 2002


Garvan Institute of Medical Research
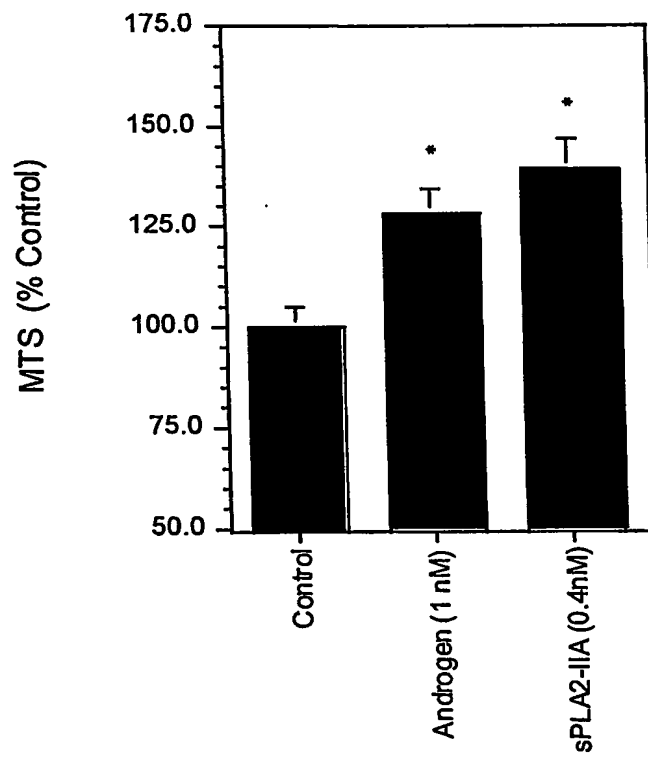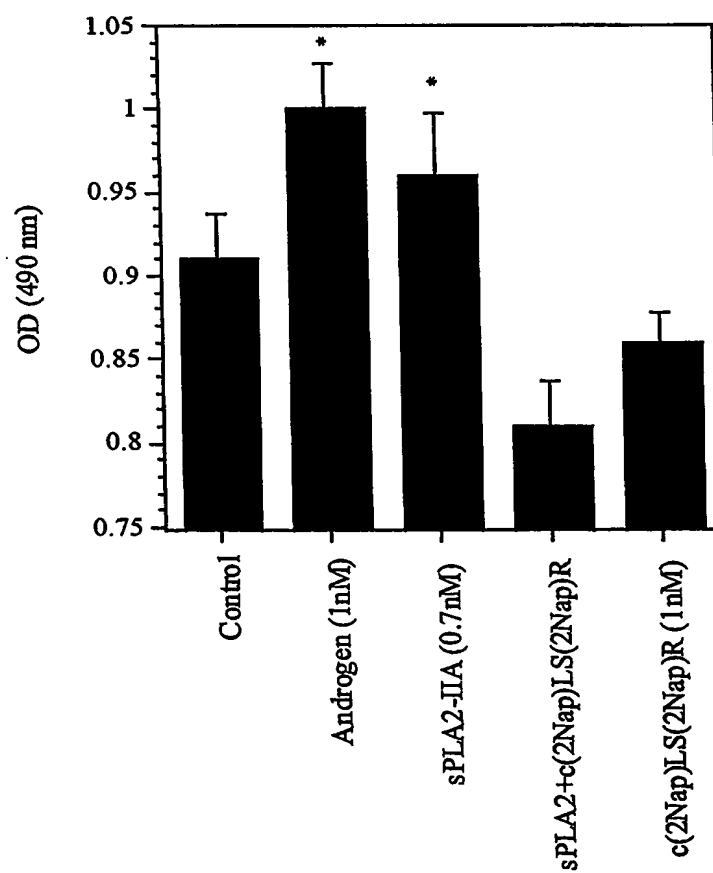Patent Attorneys for the Applicant:


F B RICE & CO

**Figure 1**

**Figure 2**